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Inactivation of Simian Rotavirus SA11 by Chlorine, Chlorine Dioxide, and Monochloramine

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The kinetics of inactivation of simian rotavirus SA11 by chlorine, chlorine dioxide, and monochloramine were studied at 5°C with a purified preparation of single virions and a preparation of cell-associated virions. Inactivation of the virus preparations with chlorine and chlorine dioxide was studied at pH 6 and 10. The monochloramine studies were done at pH 8. With 0.5 mg of chlorine per liter at pH 6, more than 4 logs (99.99%) of the single virions were inactivated in less than 15 s. Both virus preparations were inactivated more rapidly at pH 6 than at pH 10. With chlorine dioxide, however, the opposite was true. Both virus preparations were inactivated more rapidly at pH 10 than at pH 6. With 0.5 mg of chlorine dioxide per liter at pH 10, more than 4 logs of the single-virus preparation were inactivated in less than 15 s. The cell-associated virus was more resistant to inactivation by the three disinfectants than was the preparation of single virions. Chlorine and chlorine dioxide, each at a concentration of 0.5 mg/liter and at pH 6 and 10, respectively, inactivated 99% of both virus preparations within 4 min. Monochloramine at a concentration of 10 mg/liter and at pH 8 required more than 6 h for the same amount of inactivation.

The virus that causes human infantile gastroenteritis was first visualized by electron microscopy in 1973 (2). Because the virus had the appearance of a wheel with radiating spokes, it was named rotavirus; however, rotavirus has also been called orbivirus, duovirus, reovirus-like agent, and infantile gastroenteritis virus (21). Rotaviruses have now been classified as a separate genus within the Reoviridae family of RNA viruses (13). Several outbreaks of viral gastroenteritis have implicated water as a vehicle for virus transmission (10, 12, 18, 22), and because of certain of their physical properties, rotaviruses could be spread by this route. Among these properties are that the virus is shed at high levels (as much as 10^{10} particles per g) in feces of infected individuals and that it is apparently shed in large clumps (4, 14). Also, the virus does not adsorb very efficiently to aluminum hydroxide or activated sludge flocs (7), and it is environmentally stable in natural waters (11). Because rotaviruses may be spread through the aquatic environment, it is important to determine whether they can be removed or inactivated during water treatment processes.

Several reports in the literature have indicated that rotaviruses are not readily inactivated by chlorine and other disinfectants. Brade et al. (3) found that only two of five commercial disinfectants tested were effective for inactivation of rotavirus SA11. Snodgrass and Herring (17) found that over the course of 2 h neither a clarified lamb rotavirus preparation nor the same virus in intestinal contents was inactivated by a sodium hypochlorite solution (0.33% available chlorine). Tan and Schnagl (19) exposed both a clarified rotavirus SA11 preparation and the same virus in feces to each of several disinfectants including high and low concentrations of a sodium hypochlorite solution. Their report did not state either the chlorine residuals attained or the pH of test solutions evaluated. Seven logs of clarified virus preparation were inactivated in 15 s by the high chlorine concentration. With the low chlorine concentration, virions in a similar sample survived 2 h of contact. Their results regarding inactivation of the virus in feces were variable, probably due to chlorine demand substances present in the feces. In a reappraisal of this study, Tan and Schnagl (20) reported that the Wa strain of the human rotavirus was very sensitive to a hypochlorite-based disinfectant even in the presence of organic matter.

The purpose of the present study was to evaluate whether chlorine or suggested alternative disinfectants for water treatment inactivate rotaviruses. Simian rotavirus SA11 was selected as a model for the human rotavirus because cultures of human rotavirus which would grow to sufficiently high titers in established cell lines were not readily available at the time this study was started.

MATERIALS AND METHODS

Preparation and purification of stock virus. Simian rotavirus SA11 was obtained from Dale Van Donsel of the U.S. Food and Drug Administration, Cincinnati, Ohio. The virus had been passaged four times in BGM (African green monkey kidney) cell cultures and was subsequently subcultured an additional three times in the same cell line in our laboratory. For preparation of stock virus, BGM cells were grown in 690-cm^2 roller bottles. The cultures were washed three times with 50-ml portions of a medium containing 50% Eagle minimum essential medium in Hanks balanced salt solution supplemented with L-glutamine and nonessential amino acids (MEM) and 50% Leibovitz medium (L-15). This combined medium, designated MEM-L-15, also contained 0.15% sodium bicarbonate and certain antibiotics (100 U of penicillin, 100 μg of streptomycin, 12.5 μg of tetracyn, and 1 μg of amphotericin B per ml of completed medium). Before infection, the cultures were drained of media and then inoculated with rotavirus at an approximate multiplicity of infection of 0.4. The inoculum was spread evenly over the surface of the cell monolayer by manually rotating the bottles. An additional 50 ml of MEM-L-15 medium was then added, and the bottles were placed on a roller apparatus and incubated at 37°C. When 75 to 100% of the cells showed

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virus-induced cytopathogenic effects, the cultures were subjected to three freeze and thaw cycles. The virus preparations were pooled, sonicated at 100 W (Biosonik IV; Bronwill, VWR Scientific Inc., San Francisco, Calif.) for 1 min, and centrifuged at 1,300 × *g* for 30 min to remove cellular debris. For purification purposes, equal volumes of the viral supernatant and Freon 113 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) were mixed in a blender (The VirTis Co., Inc., Gardiner, N.Y.) at a setting of 60 for 1 min. The mixture was then centrifuged, the supernatant (upper aqueous layer) was removed by aspiration, and sufficient 0.06 M $MgCl_2$ was added to obtain a final concentration of 0.006 M. The pH was next adjusted to 3.0 to 3.1 with 1 N HCl, and the preparation was filtered through an HA membrane filter (47 mm; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) that had been previously washed with sterile distilled water. The membrane containing adsorbed virus was washed three times with three 100-ml portions of 0.006 M $MgCl_2$ at pH 3.0 to remove residual chlorine demand substances. The virus was eluted from the membrane with a volume of demand-free water (adjusted to pH 11.4) equal to 5 ml less than the starting volume of the virus preparation supernatant (before acidification). The eluted virus was collected in 5 ml of 0.05 M KH_2PO_4 buffer at pH 7. The purified virus had a negligible chlorine demand and was frozen in 2-ml portions at −70°C.

Cell-associated virus was prepared in 75-cm^2 flasks of African green monkey kidney cells. The cultures were washed three times with the MEM-L-15 medium and then inoculated with the virus at a multiplicity of infection of ca. 20. After 15 min of incubation at room temperature, 20 ml of MEM-L-15 was added to each culture; then the cultures were incubated at 37°C. When 50 to 100% of the cells showed cytopathogenic effects, the cultures were refrigerated for 30 min, and the cells were scraped from the flasks with a rubber policeman. The cellular material was centrifuged at 1,100 × *g* for 10 min. The supernatant was discarded, and the sediment was suspended in 50 ml of chlorine demand-free 0.05 M phosphate buffer, pH 7. After a second wash with the buffer, the cell-associated virus was suspended in 25 ml of buffer and refrigerated until used in experiments. The chlorine demand of this virus preparation was negligible.

Assay for virions. Virions were assayed by the plaque technique (5) in African green monkey kidney cells grown in 25-cm^2 flasks. The cultures were washed three times with MEM-L-15 medium, and then each was inoculated with 1 ml of virus sample. Dilutions of virus samples were made in the MEM-L-15 medium. After 1 h of incubation at room temperature, the inoculated cultures were overlaid with a medium consisting of Eagle MEM containing 1.5% (wt/vol) purified agar (Oxoid Ltd., London, England), 15 μg of neutral red per ml, 0.15% (wt/vol) $NaHCO_3$, 100 μg of DEAE dextran (M_W, 2 × 10^6) per ml, and 0.001 M $CaCl_2$. Pancreatin was also added to the overlay medium. To accomplish this, one buffered pancreatin tablet (Oxoid) was dissolved in 50 ml of water and filtered through a membrane (0.45-μm pore size). This pancreatin stock solution was added to the overlay medium at a concentration of 1% (wt/vol).

Stock chlorine solutions (ca. 1 mg/ml) were prepared from Clorox (The Clorox Co., Oakland, Calif.) or laboratory-grade sodium hypochlorite solution (Fisher Scientific Co., Pittsburgh, Pa.). Engelbrecht et al. (6) reported that chlorine solutions prepared from Clorox or chlorine gas gave identical inactivation curves with poliovirus 1; however, the stock chlorine solution prepared from Clorox was more stable than that prepared from chlorine gas. Chlorine concentration was determined by the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) method (1). The stock chlorine dioxide solution was prepared from the gas generated by adding dilute H_2SO_4 to a sodium chlorite solution (1), and the concentration was determined by the DPD method (1). Stock monochloramine solutions were prepared weekly by combining a chlorine solution and a solution of $(NH_4)_2SO_4$. The mixture ratio was 4 mg of ammonia to 1 mg of chlorine (as HOCl). The chlorine solution was prepared to contain 200 mg of HOCl in 100 ml of distilled water, and the pH was adjusted to 9 to 10. The $(NH_4)_2SO_4$ solution was prepared by dissolving 3.1 g of the salt in 100 ml of distilled water and adjusting the pH to 9 to 10. The two solutions were then combined, and the resulting monochloramine concentration was determined by the DPD method (1). For the inactivation experiments, the stock monochloramine solution was diluted in 0.05 M phosphate buffer (pH 8) to a concentration of ca. 10 mg/liter. The diluted monochloramine solution did not produce a color when added to the DPD reagent, indicating that no free chlorine was present. There apparently were no dichloramines present because the addition of excess KI after the monochloramine reading was recorded did not result in further color development in a 2-min period. The monochloramine solution was also free of nitrogen trichloride (1).

Inactivation test procedure. The pH of the buffer (0.05 M KH_2PO_4) used for the pH 6 and 8 studies was adjusted to the desired pH level with 10 M NaOH and then made chlorine demand-free by adjusting it to a free-chlorine concentration of 1 to 2 mg/liter. The buffer was kept at a room temperature for 1 or 2 days, then dechlorinated by boiling for 5 min, and then cooled and exposed to UV radiation (254 nm) for 24 to 48 h. When no free chlorine was detected in a spot test with orthotolidine, the buffer was considered demand-free. Boric acid buffer (0.05 M) was prepared from boric acid. It was made demand-free in the same manner as the phosphate buffer with the exception that the pH was adjusted after the solution had been made demand-free. The boric acid buffer was used only for experiments at pH 10. For test purposes, quantities of the appropriate stock disinfectant were added to the demand-free buffer. When the desired disinfectant concentration was obtained in the buffer, disinfectant-supplemented or unsupplemented buffer (400 ml) was placed in beakers. The beakers and remaining buffer were refrigerated overnight. The disinfectant concentration was determined the following day, and the concentration was readjusted when necessary. A multiple stirring device (Phipps and Bird, Inc., Richmond, Va.) equipped with stainless-steel paddles and operated at full power was used for stirring the samples during the inactivation experiments. Each stirring paddle was temporarily immersed in the refrigerated solution that would subsequently be stirred by that paddle. The buffer in each beaker was then discarded, and 400 ml of the remaining refrigerated buffer was added. The beaker was placed in a 5°C water bath, and after the temperature had equilibrated, stirring was started, and 1 ml of the desired test virus suspension was added. At timed intervals, samples were pipetted into a 0.05 M (pH 7) phosphate buffer neutralizing solution which contained 1.25 mg of sodium thiosulfate per ml and then assayed for virus. The residual disinfectant concentration was also determined at timed intervals. Control samples consisted of buffer supplemented with disinfectant that had been neutralized and then inoculated with virus and of unsupplemented buffer inoculated with virus. The virus titers obtained from the two controls for each test were averaged, and the titer obtained was considered as 100% survival. Percent survival at each timed interval was calcu-

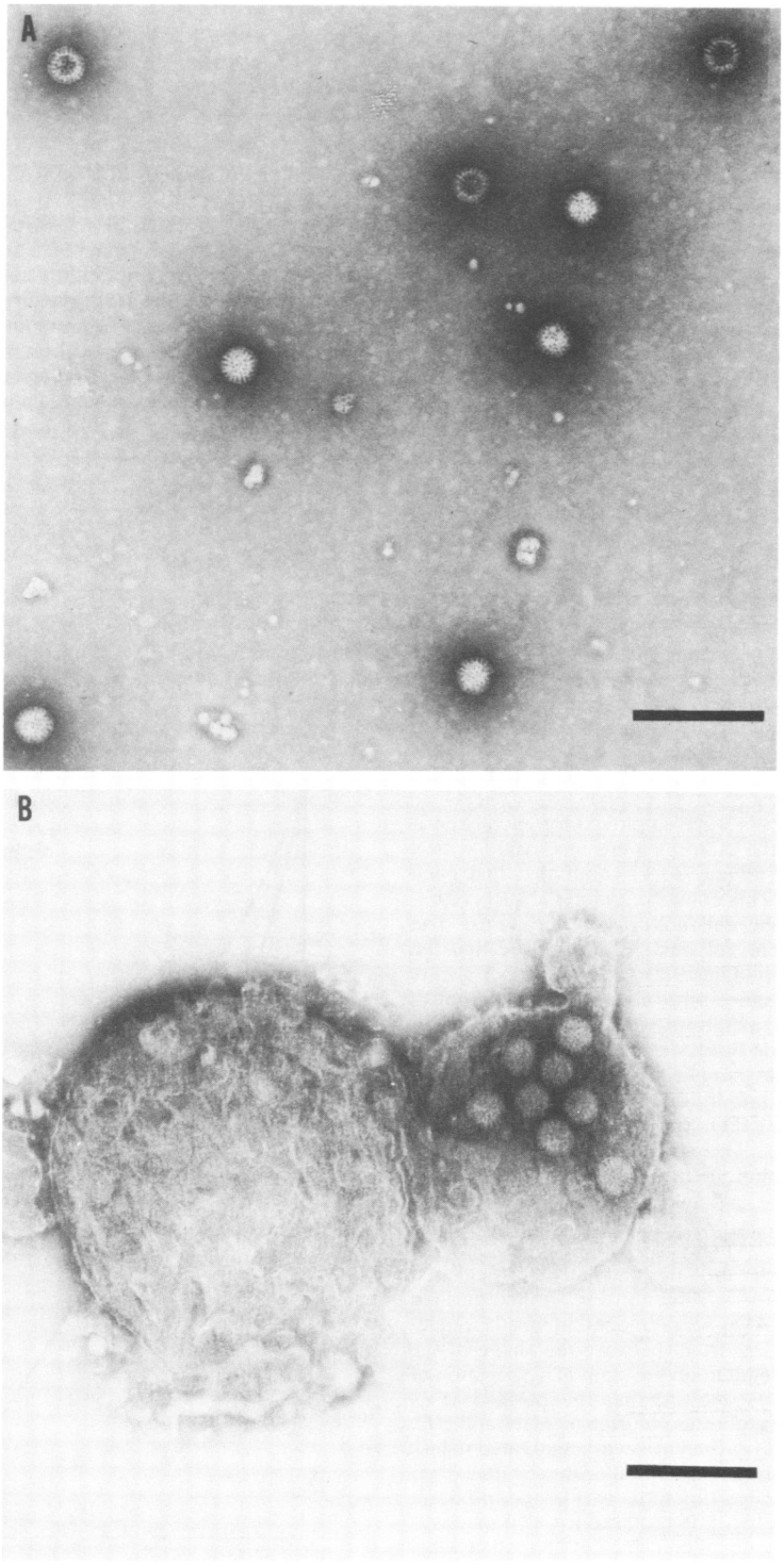

FIG. 1. Electron micrograph of purified (A) and cell-associated (B) rotavirus SA11. Bar, 250 μm.

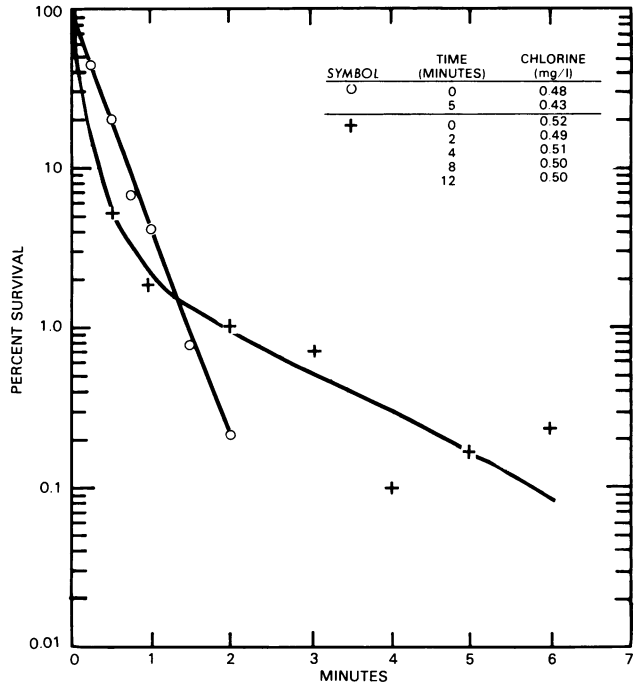

FIG. 2. Effect of 0.43 and 0.5 mg of chlorine per liter at pH 9.9 and 5°C on purified (○) and cell-associated (+) SA11.

lated, and the results (percent survival versus time) were plotted on semilog paper.

Two cell culture controls were employed for each experiment. For one control the cultures were inoculated with a stock suspension of SA11 (ca. 30 PFU/ml) that had been frozen in 2-ml portions. This control verified the susceptibility and sensitivity of the cell cultures to the virus. The purpose of the second control was to demonstrate that the cell cultures contained no indigenous viruses or other agents which might appear to cause viral plaques. To accomplish this purpose, uninoculated cultures were overlaid and examined daily for plaques or other agents. A chlorine quality assurance check sample supplied by the Environmental Monitoring and Support Laboratory of the U.S Environmental Protection Agency (EPA), Cincinnati, Ohio, was also tested on a routine basis. The assay of this sample demonstrated that our spectrophotometer and reagents were giving accurate assay values.

RESULTS

An electron micrograph of the purified and cell-associated preparations of SA11 virus is shown in Fig. 1. The purified virus preparation (Fig. 1A) appears to contain mostly single-virion particles. This is important because the shape of the inactivation curve is dependent on the state of aggregation of the virus. Repeated tests showed that the purified virus preparation was very sensitive to chlorine at pH 6 and 5°C. More than 4 logs (99.99%) of the virus was inactivated in less than 15 s by 0.5, 0.25, and 0.1 mg of chlorine per liter (data not shown). However, when this virus was tested in borate buffer at pH 10 and 5°C, the time required for 99% inactivation of the virus with 0.43 mg of chlorine per liter was ca. 1.4 min (Fig. 2).

The inactivation of SA11 by 0.5 and 1 mg of ClO_2 per liter at pH 6 and 5°C is shown in Fig. 3. The times required for 99% inactivation of the virus were 12 and 23 s for 1.0 and 0.5 mg of ClO_2 per liter, respectively. Thus, chlorine dioxide at pH 6 did not inactivate SA11 as rapidly as did chlorine. At pH 10, however, 0.5 mg of chlorine dioxide per liter inactivated more than 99.99% of the virus in less than 15 s (data not shown).

The purified SA11 virus was apparently resistant to 10 mg of monochloramine per liter at pH 8 and 5°C. The virus was 99% inactivated in ca. 6.75 h (Fig. 4). With the cell-associated virus preparation, 99% inactivation required an average time of 10.2 h (range, 9.5 to 10.6 h) (Fig. 4).

The electron micrograph of the cell-associated preparation of SA11 (Fig. 1B) shows that the virus and the cellular debris appear to be present in large clumps. Inactivation experiments with this virus preparation showed that inactivation occurred at a slower rate than with the purified virus preparation. A series of seven experiments with cell-associated virus and 0.5 mg of chlorine per liter at pH 6 and 5°C gave an average time of 1.75 min for 99% inactivation of the virus (range, 1.10 to 2.38 min). A typical inactivation curve with this virus is shown in Fig. 5. At pH 10, the average time for 99% inactivation of the cell-associated virus with 0.5 mg of chlorine per liter was 3.5 min (range, 2.5 to 4.4 min) (Fig. 2).

The inactivation of the cell-associated SA11 virus preparation by 0.5 mg of chlorine dioxide per liter at 5°C and pH 6 and 10 is shown in Fig. 6. At pH 6, the average time for 99% inactivation of the virus was 2.9 min with a range of 1.9 to 4.7

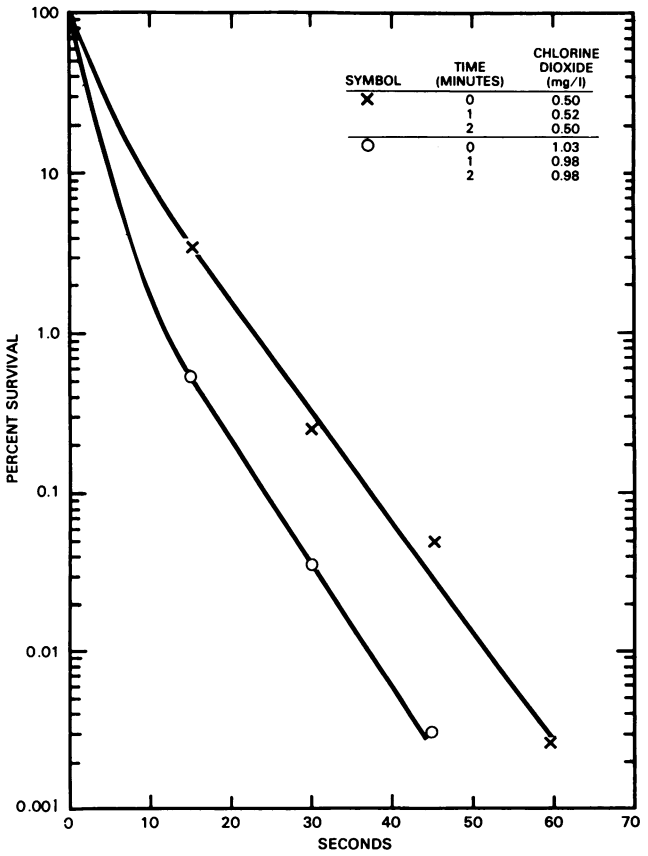

FIG. 3. Effect of 0.5 and 1.0 mg of chlorine dioxide per liter at pH 6 and 5°C on purified SA11.

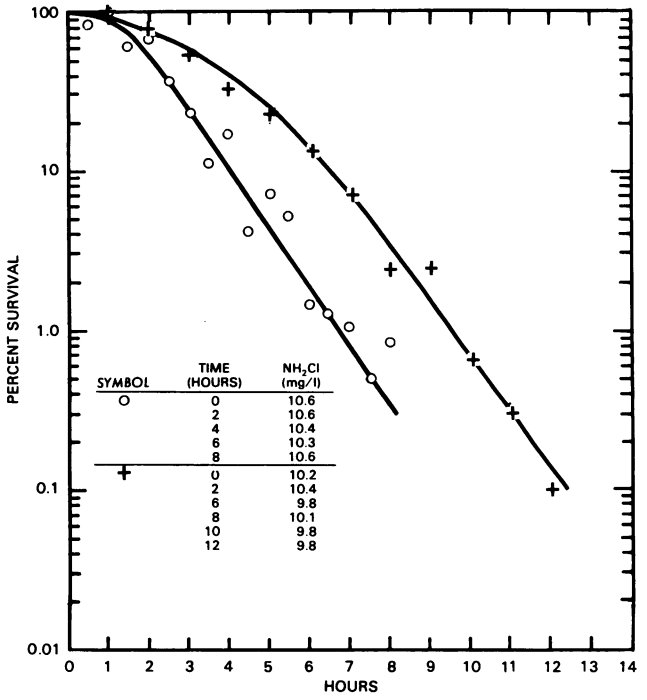

FIG. 4. Effect of 10.6 and 9.8 mg of monochloramine per liter at pH 8 and 5°C on purified (○) and cell-associated (+) SA11.

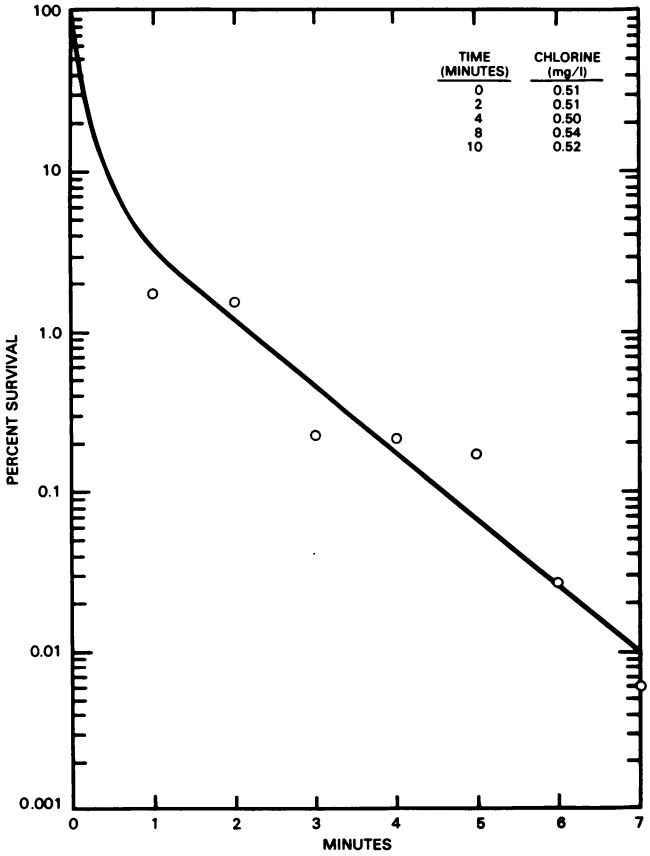

FIG. 5. Effect of 0.52 mg of chlorine per liter at pH 6 and 5°C on cell-associated SA11.

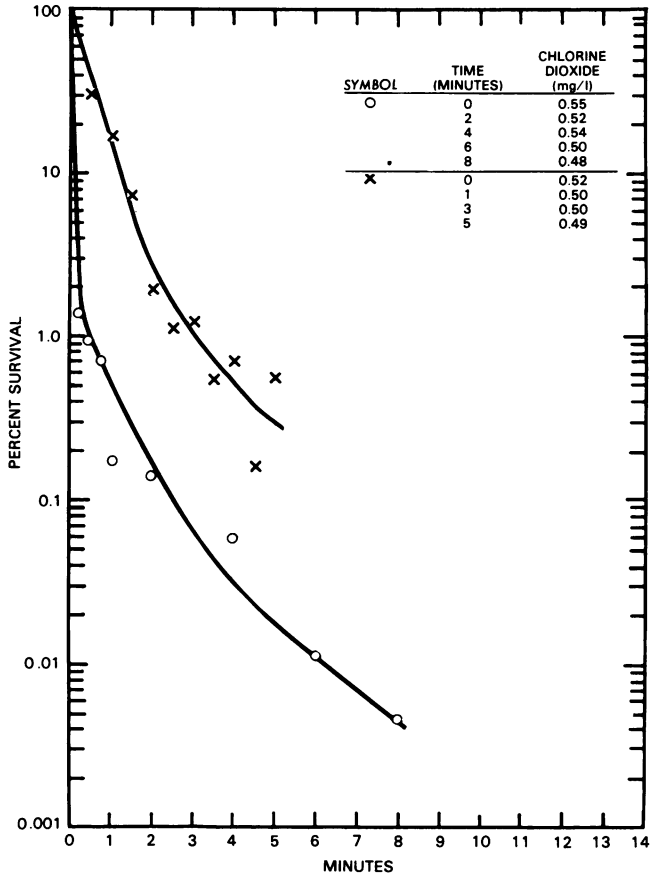

FIG. 6. Effect of 0.48 mg of chlorine dioxide per liter at pH 6 (×) and pH 9.9 (○) at 5°C on cell-associated SA11.

min. The time for 99% inactivation at pH 10 was about 0.4 min (range, 0.3 to 0.42 min).

The times required for 99% inactivation of the purified and cell-associated virus by the three disinfectants are summarized in Tables 1, 2, and 3.

DISCUSSION

Early literature reports (17, 19) indicated that rotaviruses were resistant to common disinfectants, including chlorine. However, these experiments were not done with purified virus preparations and were not adequately controlled. For example, in one report the virus was diluted in Tris buffer. In our laboratory a 0.01 M solution of Tris had a chlorine demand of greater than 20 mg/liter. Our results show that

TABLE 1. Time required for 99% inactivation of SA11 by chlorine at 5°C

Virus	pH	Concn of chlorine (mg/liter)	Time for 99% inactivation (min)
Purified SA11 (single virions)	6	0.11–0.67	<0.25
	10	0.42–0.49	1.4
Cell-associated SA11	6	0.48–0.53	1.75
	10	0.49–0.56	3.5

purified simian rotavirus SA11 is very sensitive to free chlorine at pH 6 and 5°C. The inactivation rate was too rapid to measure even at 0.1 mg of chlorine per liter. In one experiment with a 0.5-mg/liter concentration of chlorine, no viruses survived a 5-s contact time. A fast sampling apparatus described by Sharp et al. (16) might be helpful to study this inactivation rate. Because the pH of the disinfectant solution has an effect on the species of chlorine present and an effect on the virus as well, inactivation experiments were also conducted at pH 10. Even though virus survival time was greater at pH 10, 99% of SA11 was inactivated by 0.5 mg of chlorine per liter in less than 2 min (Fig. 2). The borate buffer used for the pH 10 experiments did not contain KCl because of the effect of KCl on the inactivation rate (6, 15). Because free chlorine could not be determined at pH 10 by the DPD method, the 10-ml sample taken for chlorine analysis was added to 0.14 ml of a 1:10 dilution of concentrated H_2SO_4. The final pH was ca. 6. To determine the effect of this modification of the DPD method on the chlorine analysis, we chlorinated equal volumes of chlorine demand-free buffer at pH 6 and 10 with the same volume of a stock chlorine solution. Upon analysis, the concentration of chlorine was found to be the same in the pH 10 buffer (modified DPD procedure) as it was in the pH 6 buffer (unmodified DPD procedure).

Chlorine dioxide is also an effective disinfectant against the purified SA11 virus. More than 99% of the virus was inactivated by 0.5 mg of ClO_2 per liter at pH 6 in less than 1 min. At pH 10, the rate of inactivation with 0.5 mg of ClO_2 per liter was too rapid to measure. More than 99% of the virus was inactivated in less than 15 s. Thus, the virus inactivation rate increased with an increase in pH from 6 to 10. With chlorine, the opposite was true, namely that the virus inactivation rate decreased with an increase in pH from 6 to 10.

Electron micrographs of stools containing rotavirus (14) show that the virus can be excreted in very large clumps; in this form the virus could be resistant to inactivation. Hoff (9) reported that cell-associated poliovirus was more resistant to chlorine than freely suspended virions. We found this to be true with the SA11 virus also. The effect of pH on the virus inactivation rate was also evident with the cell-associated virus preparation. Thus, the virus inactivation rate with chlorine decreased with an increase of pH from 6 to 10. With ClO_2 the inactivation rate increased with an increase of pH from 6 to 10. The cell-associated rotavirus was more resistant to inactivation with chlorine and chlorine dioxide at pH 6 and 10 than was the purified SA11 virus preparation.

Monochloramine that was shown not to be contaminated with either free chlorine or dichloramine was found to be a slow-acting viricide for purified and cell-associated SA11. The time for 99% inactivation of both virus preparations was measured in hours (Table 3). It should be noted that these inactivation curves were obtained with a relatively high concentration of monochloramine (ca. 10 mg/liter). Two additional controls were added during these tests. One control contained the $(NH_4)_2SO_4$ solution added to distilled water and then diluted for test purposes as if it contained monochloramine. This solution did not inactivate the SA11 virus, indicating that the inactivation obtained with monochloramine was due to the monochloramine and not to unreacted $(NH_4)_2SO_4$. The second control was used for the studies with the cell-associated virus and consisted of the test monochloramine solution inoculated with the purified SA11 preparation. This biological control would indicate whether free chlorine was present in the test monochloramine solution because that virus preparation is very sensitive to inactivation by free chlorine. There was virus survival for an hour and longer, indicating that no free chlorine was present.

TABLE 2. Time required for 99% inactivation of SA11 by chlorine dioxide at 5°C

Virus	pH	Concn of chlorine dioxide (mg/liter)	Time for 99% inactivation (min)
Purified SA11 (single virions)	6	0.49–0.54	0.56
	10	0.48–0.52	<0.25
Cell-associated SA11	6	0.45–0.49	2.9
	10	0.46–0.52	0.4

TABLE 3. Time required for 99% inactivation of SA11 by monochloramine at pH 8 and 5°C

Virus	Concn of monochloramine (mg/liter)	Time for 99% inactivation (h)
Purified SA11 (single virions)	9.98–10.6	6.75
Cell-associated SA11	9.78–10.28	10.2

The results of these studies compare favorably with the results reported by Engelbrecht et al. (6) on the inactivation of several enteroviruses with chlorine. They reported inactivation times of 0.3 and 1.5 min for 99% destruction of coxsackievirus A9 by 0.5 mg of chlorine per liter at 5°C and at pH 6 and 10, respectively. Our results with the purified preparation of the SA11 virus showed inactivation times of <0.25 and 1.4 min for 99% destruction of SA11 by 0.5 mg of chlorine per liter at pH 6 and 10, respectively. Cell-associated virus required 1.75 and 3.5 min for 99% destruction at pH 6 and 10, respectively.

The rapid inactivation of simian rotavirus SA11 by chlorine and chlorine dioxide indicates that this rotavirus should pose no problem in the disinfection process used in water supply operations. The results of the monochloramine studies indicate that at the levels used in water treatment (1 to 3 mg/liter), extremely long contact times may be needed to ensure inactivation of this virus. It should be remembered, however, that the SA11 virus was used only as a model for the human rotavirus. Now that the human rotavirus can be grown to high titer in cell cultures (8), the resistance of this virus to chlorine and other disinfectants will be studied.

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